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Improved reactive aldehyde, salt and cadmium tolerance of transgenic barley due to the expression of aldo-keto reductase genes

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Abstract

Under various stress conditions, plant cells are exposed to oxidative damage which triggers lipid peroxidation. Lipid peroxide breakdown products include protein crosslinking reactive aldehydes. These are highly damaging to living cells. Stress-protective aldo-keto reductase (AKR) enzymes are able to recognise and modify these molecules, reducing their toxicity. AKRs not only modify reactive aldehydes but may synthesize osmoprotective sugar alcohols as well. The role of these mixed function enzymes was investigated under direct reactive aldehyde, heavy metal and salt stress conditions. Transgenic barley (*Hordeum vulgare* L.) plants constitutively expressing AKR enzymes derived from either thale cress (*Arabidopsis thaliana*) (AKR4C9) or alfalfa (*Medicago sativa)* (MsALR) were studied. Not only AKR4C9 but MsALR expressing plants were also found to produce more sorbitol than the non-transgenic (WT) barley. Salinity tolerance of genetically modified (GM) plants improved, presumably as a consequence of the enhanced sorbitol content. The MsALR enzyme expressing line (called 51) exhibited almost no symptoms of salt stress. Furthermore, both transgenes were shown to increase reactive aldehyde (glutaraldehyde) tolerance. Transgenic plants also exhibited better cadmium tolerance compared to WT, which was considered to be an effect of the reduction of reactive aldehyde molecules. Transgenic barley expressing either thale cress or alfalfa derived enzyme showed improved heavy metal and salt tolerance. Both can be explained by higher detoxifying and sugar alcohol producing activity. Based on the presented data, we consider AKRs as very effective stress-protective enzymes and their genes provide promising tools in the improvement of crops through gene technology.

Key words: transgenic plant, lipid peroxidation, sorbitol, heavy metal, salt stress

Abbreviations

AKR aldo-keto reductase

ANOVA analysis of variance

4-HNE 4-hydroxynonenal

HPLC-RID high-performance liquid chromatography with refractive index detector

FWT fresh weight

GM genetically modified

MDA malondialdehyde

MG methylglyoxal

MsALR *Medicago sativa* aldose reductase

ROS reactive oxygen species

TAIR The Arabidopsis information Resource

TBARS thiobarbituric acid reactive substances

WT wild type

Introduction

The most limiting factors for plant growth and agricultural productivity are environmental stresses. In higher plants, the appearance of abiotic stresses (such as drought, salt and oxidative-stress as well as heavy metal toxicity) are connected to a series of gene expression and metabolic events and also result in decreased photosynthetic yield (Yamauchi et al. 2010). Salinity, for example, deteriorates plant health by reducing the ability of plants to take up water. This generates a water deficit that leads to the formation of reactive oxygen species (ROS) (Parida and Das 2005). Salt can also rise to toxic levels in the older transpiring leaves,

causing osmotic stress and premature senescence (Munns 2002). The heavy metal cadmium (Cd) has multiple toxic effects on plant health since it is able to bind to the sulfhydryl groups of the proteins which lead to misfolding (DalCorso et al. 2008). Due to its chemical similarity, Cd^{2+} is able to displace other essential metal ions such as Zn^{2+} and Fe^{2+}. The release of "free" ions might trigger oxidative injuries via free Fe/Cu-catalysed Fenton reactions (Polle and Schützendübel 2004). Either of these two or any other abiotic stress results in the increase of ROS at the cellular level (Smirnoff 1993). ROS are toxic products of abiotic stress with the potential to react with all biological macromolecules including lipids, proteins, and DNA. This may result in oxidative damage to cellular functions. As protection against such damages, plant cells contain a wide variety of antioxidants and antioxidant systems, capable of ROS elimination (reviewed by e.g. Mittler 2002). Lipid peroxidation, caused by membrane lipid interactions with ROS, however, may lead to further adverse effects. Hence certain products of these reactions such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) aldehydes contain two double bonds and are able to cross-link proteins (Zarkovic 2003). One possible way to defend the function of the proteins is through chaperones. In mammals, for example, 4-HNE treatment reportedly leads to increased expression of chaperones (Leonarduzzi et al. 2000). Chaperones were also suggested to be involved in cadmium and salt stress responses of plants (Suzuki et al. 2001; Song et al. 2011). Obviously, the other way to protect the cell from these oxidative injuries is the specific detoxification of the cytotoxic aldehydes derived from either glycolysis- or lipid-peroxidation. Many effective defence mechanisms have evolved for detoxification. It includes the glyoxalase system (Veena et al. 1999), glutathione-S-transferase (He et al. 1999), alkenal reductases (Mano et al. 2005), aldehyde dehydrogenases (Sunkar et al. 2003) and aldo-keto reductases (Oberschall et al. 2000). The expression level of glutathione-S-transferase has been increased in Arabidopsis as the result of MDA treatment (Weber et al. 2004). As it was mentioned above, certain

members of the aldo-keto reductase (AKR) enzyme family took part in the removal of reactive aldehyde molecules. Enzymes belonging to this superfamily are NAD(P)H-dependent with a relatively broad substrate specificity (Davidson et al. 1978; Jez et al. 1997). Their ability to accept various substrates is linked to their function in the protection against different stresses. Most AKRs fold into a typical $(\alpha/\beta)_8$-barrel structure and can be clustered into 15 families containing multiple subfamilies. The majority of the plant AKRs belongs to the AKR4 family; mostly to the AKR4C subfamily. The function of plant AKRs has recently been reviewed (Sengupta et al. 2015). The authors have classified the plant AKRs into four functional groups: Group I: Reactive aldehyde detoxification, Group II: Osmolyte production, Group III: Secondary metabolism and Group IV: Membrane transport. They underlined the role of plant AKRs e.g. in the production of commercially important secondary metabolites, in iron acquisition from the soil and in plant–microbe interactions, but most importantly the pivotal role of AKRs in plant defence mechanism. These enzymes have the ability to confer multiple stress tolerance in the plants. Stress responsive AKRs have also been predicted in 40 species based on homology to AKR4C9, one of the AKRs involved in this work (Sengupta et al. 2015). AKRs may provide stress protection basically by two ways, by producing osmolytes and/or by the detoxification of reactive aldehydes. The first isolated plant AKR, the barley aldose reductase has been shown to provide osmoprotection during embryo development (Bartels et al. 1991; Roncarati et al. 1995). Similar proteins were found in wild oat (*Avena fatua*) (AKR4C3) (Li et al. 1995) and in *Xerophyta viscosa* (AKR4C4) (Mundree et al. 1995). The desiccation-protecting properties of the AKR4C1–AKR4C4 enzymes were manifested through the production of osmolytes (such as sorbitol) and thus had the ability to protect the cellular components in the maintenance of integrity under reduced water contents (Bartels et al. 1991; Mundree et al. 1995). The dicot AKR4C enzymes, especially the alfalfa (*Medicago sativa)* MsALR (X97606) (Oberschall et al. 2000) and AKR4C9 (*At2g37770.2*)

from thale cress (*Arabidopsis thaliana*) (Simpson et al. 1999) can metabolize reactive aldehydes beside sugars. These dicot AKR4Cs protect the plants by detoxifying the oxidative stress-linked toxic aldehydes, such as malondialdehyde (MDA). A set of experimental data proved that transgenic tobacco plants, over-expressing the alfalfa *MsALR* gene were more tolerant to drought- and oxidative-stress conditions induced by methylviologen (MV), heavy metals and ultraviolet-B irradiation (Oberschall et al. 2000; Hideg et al. 2003; Hegedűs et al. 2004). Transgenic wheat plants ectopically over-expressing MsALR enzyme also showed enhanced drought tolerance as indicated by more biomass production, including more seed weight per plant (Fehér-Juhász et al. 2014). Clearing the toxic lipid peroxide degradation products, 4-hydroxynon-2-enal (HNE) and both MDA and methylglyoxal (MG) from stressed cells has been suggested as the role of alfalfa ALR (MsALR) and rice OsAKR1 proteins (Oberschall et al. 2000; Turóczy et al. 2011). The *Arabidopsis* enzyme, AKR4C9, reportedly improves the reactive aldehyde- and freezing-stress tolerance of transgenic barley (Éva et al. 2014a, b). Although improved cadmium tolerance of transgenic plants has already been observed, indicated by the low level of lipid peroxidation products, but has not yet been studied in detail (Éva et al. 2014a).

Barley is an important and major crop grown worldwide. It was one of the first food crops domesticated by humans. The breeding strategy for barley is targeted at enhancing stress tolerance and creating plants resistant to various effects of highly diverse environments. Our approach is based on gene technology. The MsALR and AKR4C9 over-expressing transgenic barley plants involved in this study have already been characterised at the DNA and protein levels (Éva et al. 2014a; Nagy et al. 2011). In this project reactive aldehyde-, salt-, and cadmium-tolerance of the proposed transgenic lines were analysed in detail. Functional

characterisation and comparison of the *Arabidopsis thaliana* AKR4C9 and the alfalfa MsALR enzymes were performed to determine their role in plant's defence against abiotic stresses.

Materials and Method

Chemicals

All chemicals were ordered from Sigma-Aldrich Ltd, Germany, unless otherwise indicated. The glutaraldehyde solution [25% (v/v)] was purchased from REANAL Ltd, Hungary.

Plant material and growth conditions

Transgenic barley (*Hordeum vulgare* L. var. Golden promise) plants over-expressing the 6xHis-tag fusion Arabidopsis AKR4C9 and 6xHis-tag fusion *Medicago sativa* MsALR enzymes have previously been produced and characterized (Éva et al. 2014a; Nagy et al. 2011). Both genes were regulated by constitutive promoters. The gene for AKR4C9 was driven by the rice actin promoter while the gene for MsALR were under the control of the maize ubiquitin promoter. Two transgenic lines were chosen from each transformation experiment in this study. Lines expressing AKR4C9 protein were called C1, C2 and C3, while 30 and 51 carrying the gene coding for the MsALR protein. None of the studied transgenic lines showed any phenotypic differences from the wild type, under non-stressed conditions.
For plant physiology experiments, both WT and transgenic barley seedlings were grown in ¼ Hoagland solution under a slightly modified protocol described by (Fodor et al. 1998) with a 14 h light period of 400 µmol m^{-2} s^{-1} light intensity and 10 h dark period.

Western blot analysis

Total leaf protein was extracted from leaves of transgenic AKR4C9- and MsALR-accumulating lines (C1, C2, C3 and lines 30, 51) and purified by affinity chromatography using His-Select™ Nickel Affinity Gel according to Éva et al. (2014a). The proteins were separated using SDS-PAGE and His-tag fusion AKR-enzymes were detected by immunoblot analysis using rabbit polyclonal antibody against the 6×His-tag (Affinity Bioreagents), as it was previously described by Éva et al. (2014a).

Glutaraldehyde treatment

Glutaraldehyde treatment of transgenic plants was carried out according to the optimized protocol of Éva et al. (2014a). Eleven-day-old seedlings (3-leaf stage), were grown in hydroponics. Twenty-five ml glutaraldehyde solution [0.1% (v/v)] was sprayed to 5 plantlets and it was repeated 2 days later. Physiological parameters of the oldest leaves were measured 24 h after the last spray. Nutrient solution was changed before the first spray only.

Sugar alcohol extraction and determination

Carbohydrate extraction and determination of sorbitol contents by HPLC-RID was performed as described earlier (Éva et al. 2014b). Sorbitol content of the oldest leaves of ten days old, unstressed plants has been analysed.

Cadmium and salt treatment

After 10 days of seed germination, Cd in the form of $Cd(NO_3)_2$ was included in the hydroponics at a concentration of 10 µM for 5 days. Salt treatment was also carried out on 10-day old seedlings for 6 days. Hoagland solution was supplemented with NaCl to reach the final concentration of 175 mM. Fluorescence induction parameters were analysed and the level of photosynthetic pigments and the level of TBARS (thiobarbituric acid reactive substances) was also measured before and after the treatments.

Determination of pigment contents, PSII maximal quantum efficiency and level of TBARS

All physiological parameters have been measured on the oldest leaves of the plants. Chlorophyll was extracted from transgenic plants with 80% (v/v) acetone-water buffer (containing 5 mM Tricine, pH 7.8) and the concentration was measured by a UV-VIS spectrophotometer (Shimadzu, Japan) according to Porra et al. (1989). Fluorescence induction measurements of leaf samples were performed using a PAM 101–102–103 chlorophyll fluorometer (Walz, Effeltrich, Germany). PSII maximal quantum efficiency has been determined following the method published earlier (Genty et al. 1989). Total carotenoid contents were measured in 100% (v/v) acetone extracts by a UV-VIS spectrophotometer (Shimadzu, Japan) as it was described by Lichtenhaler (1987). The level of lipid peroxidation products [thiobarbituric acid reactive substances (TBARS)] was determined using the modified method of Dhinsa et al. (1981). According to the protocol, 50 mg of plant sample was homogenized with 450 µl 0.1% (w/v) trichloroacetic acid and centrifuged (10000 g; 10min; 4°C). A total of 250 ml of the supernatant was mixed with 1 ml TBARS-reagent [20% (w/v) trichloroacetic acid solution containing 1% (w/v) thiobarbituric acid]. The mixture was boiled for 30 min and then cooled to room temperature. Samples were measured at 532 and

600 nm (product and background, respectively) with a Shimadzu UV2101 spectrophotometer. The concentration was calculated using a molar extinction coefficient of 1.55×10^7 mM cm^{-1}.

Statistical analysis

The reported values are the means of five independent experiments. Unpaired Student's t-tests and ANOVA tests were performed on the data as statistical analysis.

Results

Western blot analysis

In order to visualise the recombinant aldo-keto reductase enzyme expression (either AKR4C9 or MsALR) and compare their levels in the transgenic lines, Western blot analysis has been carried out. Due to the His-tag fused to both proteins they were purified and detected the same way. Protein production of the studied lines is illustrated by Fig. 1. This blot reveals that line C1 contains more AKR4C9 protein than either C2 or C3 lines. For further experiments the highest (C1) and the lowest (C2) expressing lines were chosen. Regarding MsALR expressing transgenic barley lines, 30 has lower, while 51 has higher recombinant enzyme level.

Reactive aldehyde treatment of intact plants

Reactive aldehyde detoxifying capacity of both GM and WT plants was investigated by applying 0.1% (v/v) glutaraldehyde treatment. Chlorophyll contents and photosynthetic

activity of MsALR expressing lines, as well as the carotenoid contents of both AKR4C9 and MsALR accumulating lines were studied. The total carotenoid content was significantly lower ($P<0.05$) in the WT and in the low MsALR-expressing line (line 30) after the treatment. It also decreased in other transgenic lines, albeit not significantly (Fig. 2a). It should be noted that the high MsALR-expressing line 51 contained significantly ($P<0.05$) more carotenoid than the treated WT. Total chlorophyll contents also decreased significantly ($P<0.05$) as the result of glutaraldehyde treatment in both the WT and in the low MsALR-expressing line 30, but not in the high MsALR-expressing line 51 (Fig. 2b). Similarly, the PSII maximal quantum efficiencies of the wild type and line 30 significantly ($P<0.05$) decreased, while it did not change significantly in the GM barley line 51. This line significantly ($P<0.05$) differed from WT plants (Fig. 2c).

Heavy metal (cadmium) treatment

WT barley and transgenic lines expressing either Arabidopsis or alfalfa AKR-enzymes were exposed to 10 µM of cadmium. There was no significant change in total chlorophyll contents after the treatment in any of the transgenic lines, but it significantly ($P<0.05$) decreased in the WT plant (Fig. 3a). PSII maximal quantum efficiency also significantly ($P<0.05$) decreased as a result of the Cd treatment in all plants, but remained significantly ($P<0.05$) higher in transgenic lines 30, 51 and C1 compared to the WT (Fig. 3b**)**. There was a 2-3-fold increase in the level of TBARS in MsALR-expressing transgenic lines and a 4-fold increase in WT plant (Fig. 3c), confirming that Cd causes high levels of oxidative stress and lipid peroxidation. The change was significant at the 95 % level in all lines. TBARS levels were significantly ($P<0.05$) lower in line 51, than in the WT by the end of the treatment.

Sugar alcohol contents

Sorbitol concentration of the high MsALR-expressing line 51 proved to be 0.75±0.56 µmol g fresh weight $(fwt)^{-1}$ while in the low MsALR-expressing line 30 it was 0.44±0.34 µmol g fwt^{-1}. The sugar alcohol content of WT barley plant has previously been found to be 0.17±0.23 µmol g fwt^{-1} (Éva et al. 2014b). Sorbitol production was significantly higher in the high MsALR-expressing line 51 than in WT ($P<0.05$).

Salt treatment

In order to study the effect of salt stress on AKR4C9- and MsALR-expressing transgenic barley, WT and GM plants were subjected to 175 mM NaCl treatment in hydroponics for 6 days. Though the plants were stressed, their total fresh weight increased significantly ($P<0.05$) in all lines during the treatment. It should be noted, however, that the total fresh weight of lines C1, 51 and 30 were significantly ($P<0.001$) higher, than in the case of WT (Fig. 4a). The total chlorophyll content of both WT and the transgenic barley lines C1, C2 and 30 decreased as the result of the salt stress treatment (Fig. 4b), but this was significant only in the case of WT ($P<0.05$). The chlorophyll contents of line 51, on the other hand, increased, regardless of the salt treatment. It significantly ($P<0.05$) differed from the WT at the end of the treatment. The chlorophyll *a*/*b* ratio was significantly ($P<0.05$) higher in the WT barley compared to the MsALR-expressing lines (Fig. 4c). Maximal quantum efficiency of photosystem II (PSII) reaction centres showed significantly ($P<0.05$) lower level under salt stress not only in the WT but in the AKR4C9-expressing lines as well, while not in lines expressing the MsALR enzyme (Fig. 4d). It was also shown that after salt treatment, F_v/F_m value of the high AKR4C9-expressing line C1 and both MsALR-expressing lines

significantly differed from the WT. Total carotenoid contents did not change significantly in any lines (data not shown) during the treatment, however, a significant ($P<0.05$) increase in the chlorophyll/carotenoid ratio of line 51 was observed (Fig. 4e). The level of TBARS did not increase significantly in any of the lines during the salt stress (Fig. 4f). Line 51 exhibited the highest salt tolerance, showing no visible injury at all during the treatment, while the oldest leaves of WT barley were severely damaged (Fig. 5a, b).

Discussion

An artificial reactive aldehyde (glutaraldehyde) was used in *in vivo* experiments to characterise the reactive aldehyde tolerance of transgenic barley plants accumulating two types of AKR enzyme, AKR4C9 from *Arabidopsis thaliana* and MsALR from *Medicago sativa*. It has already been demonstrated that glutaraldehyde can be successfully applied to characterise the activity and thus the protective effect of the AKR4C9 enzyme *in vivo* (Éva et al. 2014a). However, neither *in vitro* nor *in vivo* activity of MsALR has been studied against glutaraldehyde, so far. In this article, by measuring total chlorophyll contents and PSII maximal quantum efficiency, MsALR-expressing transgenic lines were also found to exhibit higher glutaraldehyde tolerance compared to the control barley. In addition to the effects previously characterised, the effect on carotenoid contents has also been demonstrated. Treated WT plants contained the least carotenoid, which may indicate the lowest glutaraldehyde tolerance. Glutaraldehyde tolerance of the transgenic plants expressing different AKRs was comparable. Based on our studies, glutaraldehyde can be suggested as an effective molecule for studying the function of reactive aldehyde detoxifying enzymes *in vivo*. Glutaraldehyde tolerance can be determined by measuring a wide range of physiological

parameters including growth parameters; chlorophyll and carotenoid contents; photosynthetic activity, and respiratory rate (Éva et al. 2014a).

Because cadmium is known to cause strong oxidative stress and lipid peroxidation, our transgenic lines expressing reactive aldehyde detoxifying enzymes were also studied for Cd tolerance. As a result of the increased concentration of AKR enzymes, transgenic barley plants displayed a slight improvement in their tolerance to Cd. The role of AKR4C9 under Cd stress had only previously been indicated by the low level of lipid peroxidation products in the high AKR4C9 expressing line C1 under 10 µM Cd stress (Éva et al. 2014a). Higher Cd tolerance of this line, compared to WT, has been confirmed during this study by measuring PSII maximal quantum efficiency and chlorophyll contents. Hegedűs and co-workers (2004) published data on the effects of MsALR in transgenic tobacco and demonstrated improved Cd-tolerance attributed to the increased amount of the aldo-keto reductase enzyme. The present study confirms that this enzyme can also enhance the Cd tolerance of the monocot plant, barley. Cd tolerance of AKR4C9 and MsALR-expressing transgenic lines were comparable. It can be hypothesized that enhancing heavy metal tolerance is a common feature of reactive aldehyde detoxifying AKR enzymes. Sugar alcohols are known as antioxidants, therefore, sugar alcohol production (see below) may have also contributed to Cd tolerance. The protective effects of AKR-overproduction on transgenic barley were not as visible in cadmium treatment as it was in the case of salt stress. High concentration of salt was clearly tolerated by the high AKR-expressing lines while Cd caused serious damage. It was of no surprise, however, because AKRs clearly cannot protect the plants from all effects of cadmium, while they address both major negative effects of salinity (i.e. oxidative and osmotic stress) by producing sugar alcohols (see below). Most other efforts to improve Cd tolerance has also focused on a single aspect of it, either detoxification by overproduction of

phytochelatins (Zhu et al. 1999) or over-expression of other antioxidant enzymes (Lee et al. 2007).

The major function of stress-induced dicot AKRs was considered to be the detoxification of several reactive aldehyde compounds. It has also been suggested that the production of sugar alcohols from carbohydrates may also be part of their stress-protecting function (Gavidia et al. 2003). Sugar alcohols confer salt stress tolerance through ROS-scavenging and by acting as compatible solutes and osmoprotectants (Parida and Das 2005). Sugar alcohol production was assumed from the low enzyme activity of stress-induced AKRs found against fructose or other carbohydrates. Evidence of enzyme activity of purified AKR4C9 against fructose and enhanced sorbitol production of AKR4C9 expressing transgenic plants has already been available (Éva et al. 2014a, b). The activity of the MsALR enzyme against fructose has not been studied so far. The present work indicates that not only AKR4C9 is capable of producing sorbitol, but MsALR as well. The concentration of the produced sorbitol is in the same range as it was published earlier for mannitol in transgenic plants expressing bacterial mannitol-1-phosphate dehydrogenase (Tarczynski et al. 1992; Abebe et al. 2003). The established plant lines enhanced the salt tolerance despite the low level of mannitol produced. This positive effect was therefore attributed not to osmoprotective activity, but rather to the ROS-scavenging activity of the sugar alcohol. One should also take into account, that the mannitol-1-phosphate dehydrogenase transgene did not offer any further advantages for the plant. In contrast, the aldo-keto reductase enzymes studied in this project were even able to reduce harmful reactive aldehydes beside sugar alcohol production and, therefore, provide further benefits.

In theory, both AKR4C9 and MsALR enzymes are able to enhance salt tolerance not only because of sorbitol production, but also because of the reactive aldehydes detoxification capability. To investigate this concept, transgenic lines were exposed to high level (175 mM) of NaCl. This severe treatment was necessary to apply because Golden Promise originally exhibits a high tolerance to salt (Forster 2001). Both types of transgenic lines showed considerably good salt tolerance, but the MsALR-expressing plants performed better during salt stress treatments. Remarkably, however, both the high and low MsALR-expressing lines contained more recombinant aldo-keto reductase protein, than their respective counterparts, the high and low AKR4C9-expressing lines (see Fig. 1). It also should be noted that the high MsALR-expressing transgenic plant line 51 was the most tolerant of the lines tested. Salinity caused only small variations in chlorophyll contents, chl*a/b* ratio, and photosynthetic activity in this particular line. Moreover, stress symptoms were barely visible even on the oldest leaves. This can be explained only by sugar alcohol production, as the level of TBARS did not increase significantly in any lines during the treatment. It is interesting to note that although sorbitol contents of the high MsALR-expressing line 51 (0.75 µmol g fwt^{-1}) were similar to the high AKR4C9-expressing line (0.8 µmol g fwt^{-1}) (Éva et al. 2014b), transgenic line 51 had much higher salt tolerance. Apart from the protein level, the possible differences in the activity of these two enzymes under salt stress could be an explanation of this feature. Because aldo-keto reductase enzymes possess broad substrate specificity (Davidson et al. 1978; Jez et al. 2007) additional products may also contribute to salt stress tolerance. Another effect of salt stress was the increase of the chlorophyll/carotenoid ratio in the highly tolerant transgenic line 51. As it was published earlier carotenoid content of rosemary had increased during water stress, it was suggested that large amounts of carotenoid might contribute to the prevention of oxidative damage (Munné-Bosch et al. 1999). Regarding our findings, we

propose that in the case of salinity stress, plants with significant AKR-protection activity may not require the elevated carotenoid content.

It was demonstrated earlier that that high salt concentration induced the expression of aldo-keto reductase in *Digitalis purpurea* (Gavidia et al. 2003) as well as AKR4C9 in *Arabidopsis thaliana* (TAIR database). Furthermore, it was also revealed that dicot AKR enzymes are able to remove reactive aldehyde as primary function and also to synthesise sugar alcohol as a secondary function. This is the first report, however, to demonstrate that such enzymes can provide enhanced salt tolerance in transgenic plants. Previous studies have assessed the performance of AKRs producing sugar alcohol as a primary function. For example, either celery or peach aldo-keto reductase enzyme was ectopically over-expressed in transgenic Arabidopsis, which lead to higher sugar alcohol content and salt tolerance was also improved (Zhifang et al. 2003; Kanayama et al. 2014). It has to be noted that apart from AKR over-expression, improved salt tolerance can be based on either osmoprotection (Shekhawat et al. 2011) or on overproducing antioxidant enzymes (Hoshida et al. 2000).

In this project two AKR enzymes (AKR4C9 and MsALR), having similar functions and amino acid sequence (68.9%), were studied in transgenic plant to compare their effects on some physiological characteristics of barley. In summary, both enzymes proved to provide enhanced reactive aldehyde, heavy metal and salt tolerance during our previous and present works. It has been demonstrated that MsALR ameliorated UV-B, drought and cold tolerance (Oberschall et al. 2000; Hideg et al. 2003; Hegedűs et al. 2004) while AKR4C9 enhanced reactive aldehyde and frost-tolerance (Éva et al. 2014a, b). Based on our data, we could not find any differences between the function of the two enzymes, despite originating from

different species. Both of them are key players in plant stress response, providing partial protection against many abiotic stresses.

Author Contributions Statement

CsÉ carried out most of the works and analysis, produced plants over-expressing Arabidopsis AKR4C9, helped draft the manuscript; ÁS designed glutaraldehyde, salt and Cd treatments, helped draft the manuscript; MO carried out pigment content determination, PSII maximal quantum efficiency and level of TBARS; RTF carried out sugar alcohol extraction and determination, analysed data; BN produced plants over-expressing *Medicago sativa* MsALR, took part in data analysis; GVH participated in the design and coordination, took part in data analysis; LT conceived the study, participated in the design and coordination and helped draft the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1
Immunoblot analysis of the His-tag fusion AKR4C9 and MsALR proteins expressed in transgenic barley leaves using anti-6×His antibody. Protein production of wild type, AKR4C9-expressing lines (C1, C2, and C3) and MsALR-expressing lines (30 and 51) are shown. The size of protein ladder is given in kDa. The figure is published with kind permission of Springer Science+Business Media because part of it has already been published showing protein production of WT and lines C1, C2 and C3 (Éva et al. 2014a).

Fig. 2
Effects of 0.1% glutaraldehyde treatment after 3 days. Total carotenoid (carotenes and xantophylls C+X) contents (**a**) of WT, transgenic barley expressing high (C1) and low (C2) amounts of AKR4C9 and expressing high (51) and low (30) amounts of MsALR. Chlorophyll contents (**b**) and PSII maximal quantum efficiency (**c**) of non-transgenic and MsALR-expressing transgenic plants. Data are the means + SDs of 5 treatments. Asterisks mark data of treated plants, significantly ($P < 0.05$) different from untreated (Day 0) samples of the same genotype. Letter 'S' marks data of treated transgenic plants significantly ($P < 0.05$) different from treated wt.

Fig. 3
Effects of 10 µM $Cd(NO_3)_2$ cadmium treatment on transgenic and WT plants after 5 days. The following physiological parameters of treated plants were determined on Day5: total chlorophyll contents (**a**), PSII maximal quantum efficiency (**b**) and level of TBARS (**c**). Data are the means + SDs of 5 treatments. WT stands for wild type, C1 for transgenic line expressing high and C2 for expressing low amounts of AKR4C9. Line 51 is a transgenic line expressing high and 30 expressing low amounts of MsALR. Data are the means + SDs of 5 treatments. Asterisks mark data of treated plants, significantly ($P < 0.05$) different from untreated (Day 0) samples of the same genotype. Letter 'S' marks data of treated transgenic plants significantly ($P < 0.05$) different from treated wt.

Fig. 4
Effects of 175 mM NaCl treatment after 6 days. Total fresh weight (**a**), total chlorophyll contents (**b**), chlorophyll *a/b* ratio (**c**), PSII maximal quantum efficiency (**d**), chlorophyll/carotenoid ratio (**e**) and the level of TBARS (**f**) in WT, transgenic barley expressing high (C1) and low (C2) amounts of AKR4C9 or expressing high (51) and low (30) amounts of MsALR. Data are the means + SDs of 5 treatments. Asterisks mark data of treated plants, significantly ($P < 0.05$) different from untreated (Day 0) samples of the same genotype. Letter 's' marks data of treated transgenic plants significantly ($P < 0.05$), while s significantly ($P < 0.001$) different from treated wt.

Fig. 5
Representative photo of salt-stressed non-transgenic (WT) (**a**) and transgenic (line 51) (**b**) barley plants grown in 175 mM NaCl containing ¼ Hoagland solution after 6 days.

Figure

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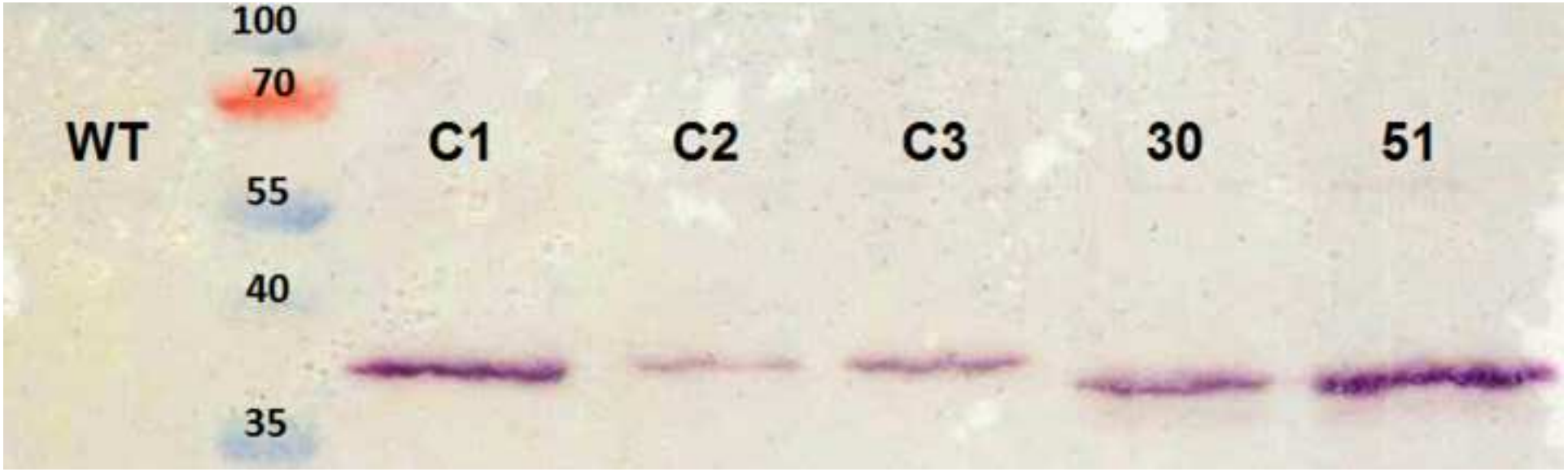

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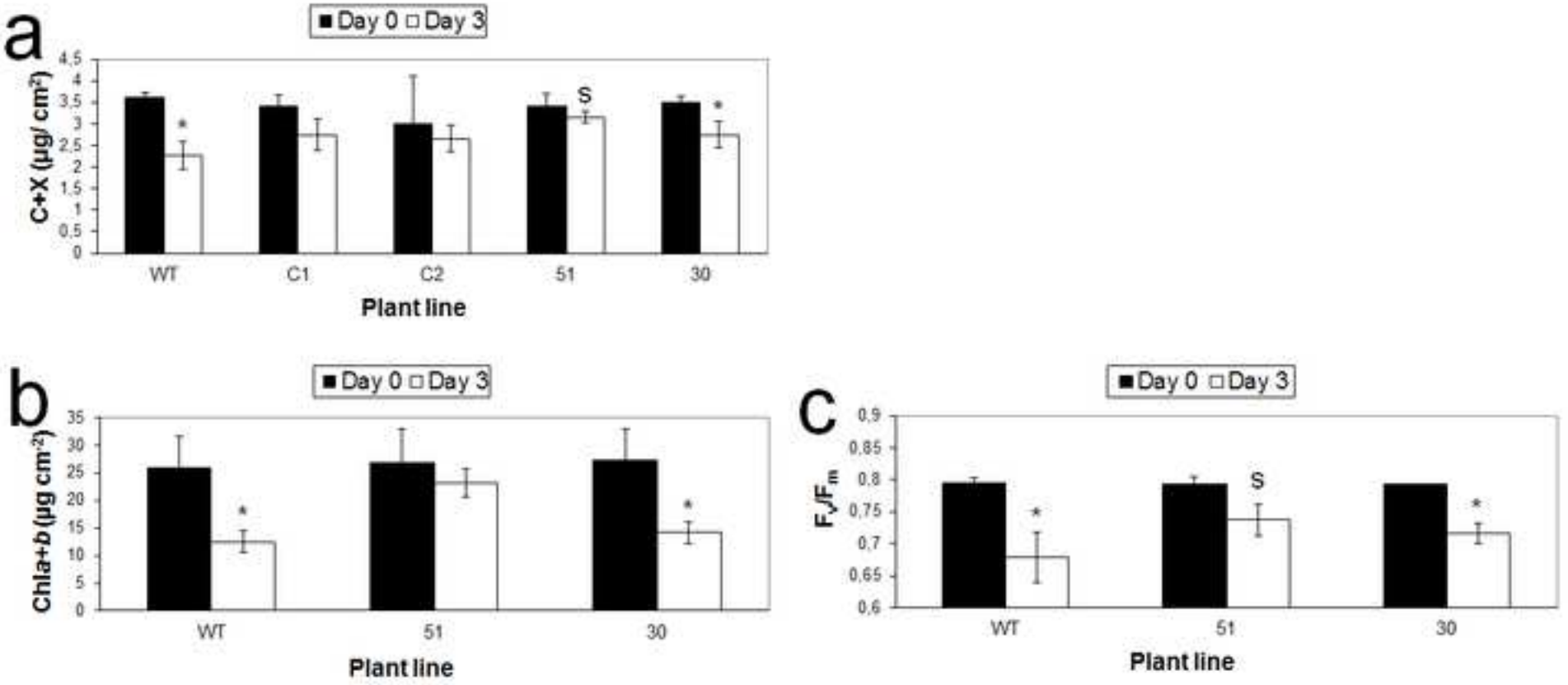

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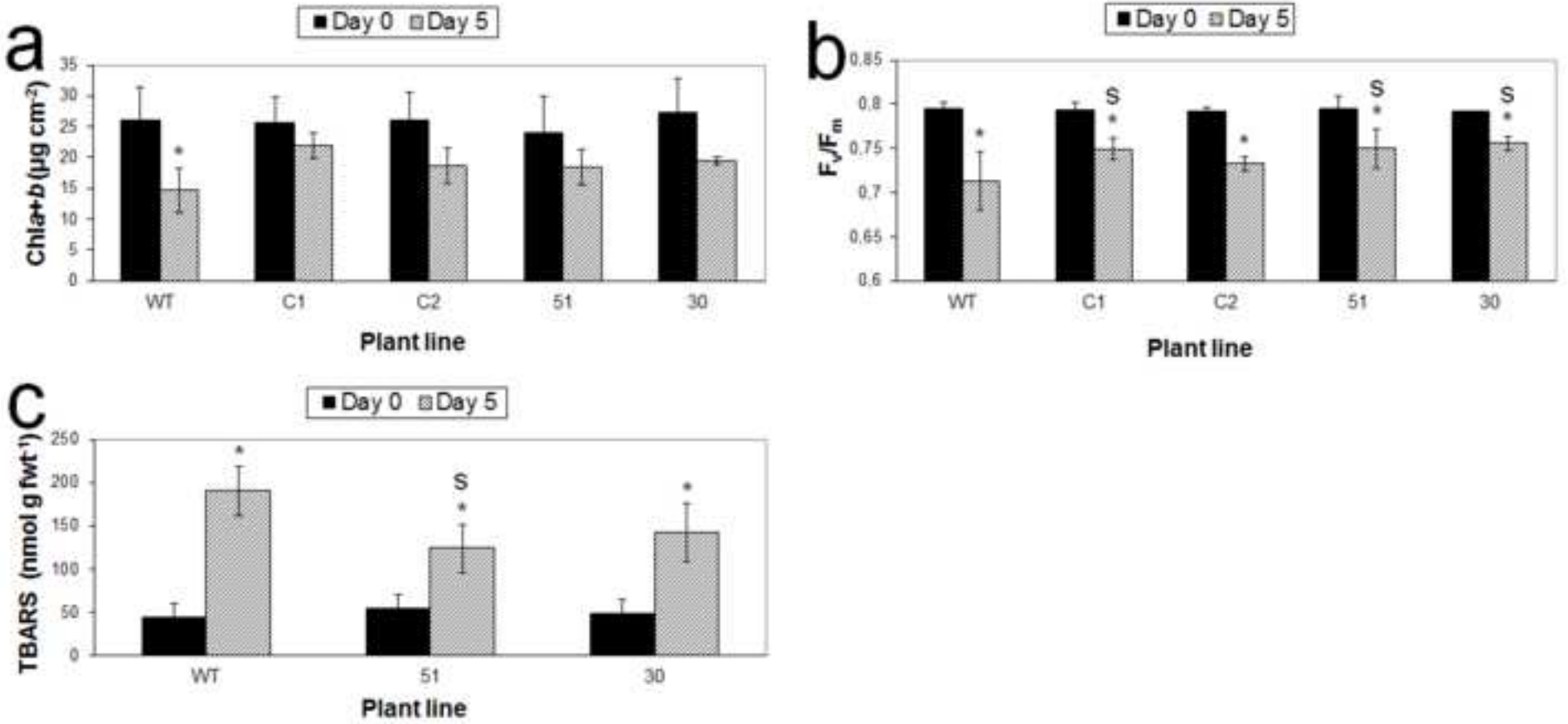

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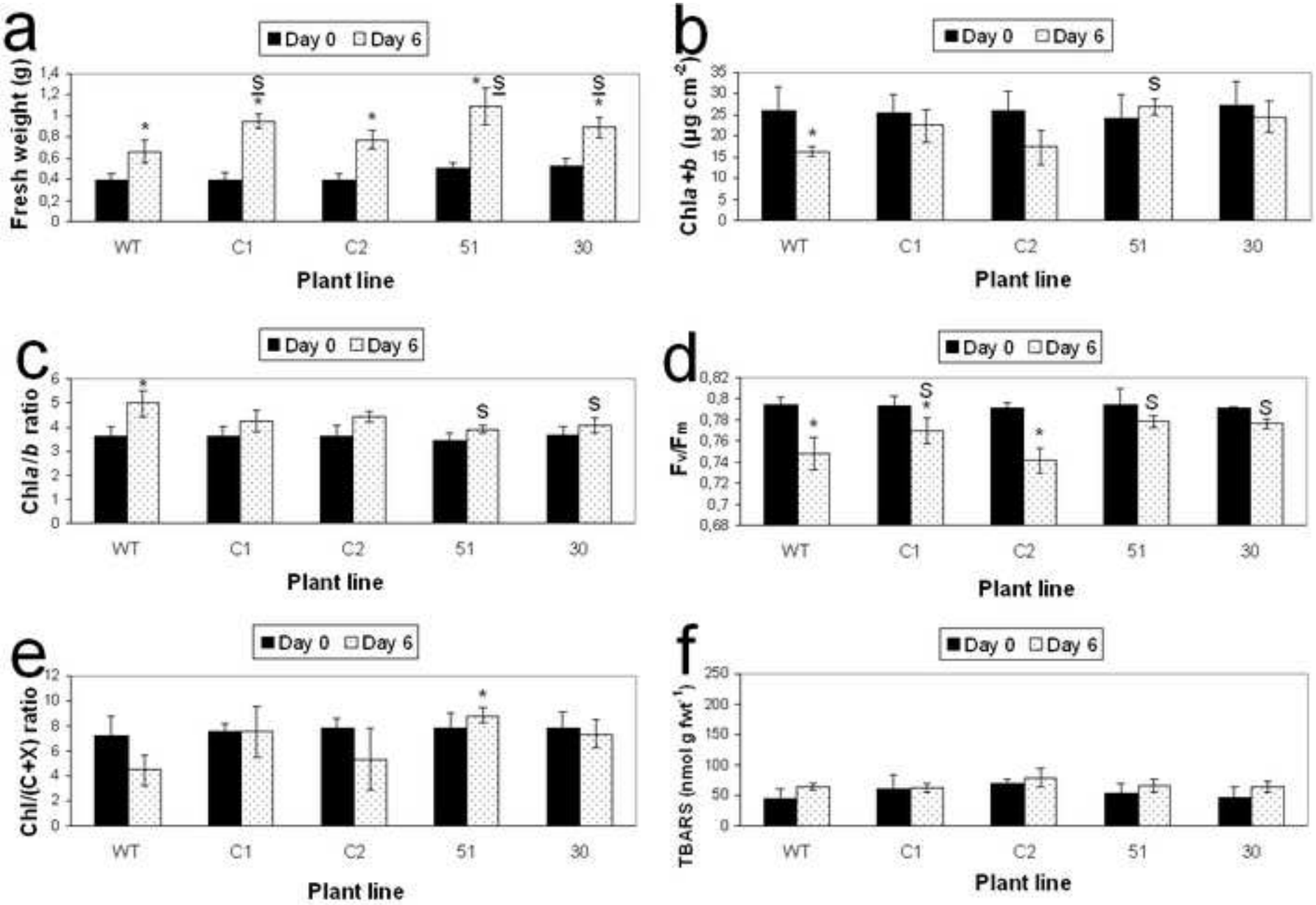

Figure

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